Nitric oxide levels produced by peritoneal macrophages in mice inoculated with *Lactobacillus casei* and infected with *Babesia microti*

Niveles de óxido nítrico producido por macrófagos peritoneales de ratones inoculados con Lactobacillus casei *e infectados con* Babesia microti

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ABSTRACT

This study was conducted to evaluate the role of nitric oxide (NO) in the innate immune response against infection with *Babesia microti*. Four groups of NIH mice (n = 6 / group) were inoculated intraperitoneally as follows: *Babesia microti* (Bm-1: 5 x 10^5 *B. microti*-infected erythrocytes/ mouse on day -1); *Lactobaciullus casei* (Lc-1 on day -1); negative control (PBS-1: PBS on day -1) and *L. casei* with *B. microti* (Lc-1 Bm-0) inoculated with lactobacilli on day -1 and *B. microti* on day 0. On day 0, peritoneal macrophages were obtained, adjusted to 1x10^6 cells/ml and incubated at 37 °C in the presence of 5% CO_2 for 24 h; each supernatant from each well was collected and the NO was determined. The average NO values obtained were the following: 11 904.63 ng/ml for the Bm-1 group and 4 520.61 ng/ml for the Lc-1 group (P <0.0001). The Lc-1 Bm-0 group had an average of 1 237.22 ng/ml which was lower than that observed in Lc-1 group (P <0.01). Parasites did not establish in Lc-1 Bm-0 group, which suggests that the NO induced in macrophages by *L. casei* was used to control the *B. microti* infection. It is concluded that *L. casei* stimulates NO production in mouse peritoneal macrophages and that NO levels diminish when these cells are confronted with the protozoan pathogen *B. microti*.
Key words: babesiosis, nitric oxide, mice.

RESUMEN

El presente estudio se llevó a cabo con objeto de evaluar la participación del óxido nítrico (ON) en la respuesta inmune innata contra la infección por *Babesia microti*. Cuatro grupos de ratones NIH (n=6/grupo) fueron tratados por vía intraperitoneal de la siguiente manera: *Babesia microti* (Bm-1: 5 x 10^5 eritrocitos infectados con *B. microti*/ratón en el día -1), *Lactobaciullus casei* (Lc -1 en el día -1), Testigo negativo (PBS -1: PBS en el día -1) y *L. casei* con *B. microti* (Lc-2 Bm-1: lactobacilos en el día -1 y *B. microti* en el día 0). En el día 0, se procedió a obtener macrófagos peritoneales, se ajustó a 1x10^6 células/ml que se incubaron a 37 °C en presencia de 5% CO_2 durante 24 h; el sobrenadante de c/pozo se recolectó y se le determinó ON en ng/ml. Los promedios obtenidos fueron: 11 904.63 ng/ml para el grupo Bm-1 y de 4 520.61 ng/ml para el grupo Lc-1 (P < 0.0001). El grupo Lc-1 Bm-0 presentó un promedio de 1 237.22 ng/ml que fue menor (P<0.01) a lo observado en el grupo Lc-1. Los parásitos no se establecieron en el grupo Lc-1 Bm-0, lo que sugiere que el ON inducido por *L. casei* en macrófagos, fue utilizado para controlar la infección por *B. microti*. Se concluye que *L. casei* estimula la producción de ON en macrófagos peritoneales de ratón y que los niveles de éste disminuyen cuando se confrontan dichas células con el protozoario patógeno *B. microti*.
Palabras clave: babesiosis, óxido nítrico, ratones

INTRODUCTION

Previous studies showed that *Lactobacillus casei* stimulates the innate immune system resulting in protection against various parasites (Bautista & Figueroa, 2004) including *Babesia microti* in mice (Bautista et al., 2005). In studies carried out against this pathogen it was suggested that the *Lactobacillus* stimulated production of nitric oxide (NO). NO is an oxidant which has a pair of unpaired electrons, which is not considered as an oxidizing free radical; however, it reacts with molecular O_2 and produces H_2O_2, among other highly toxic and reactive varieties. (Pozo et al., 1988)

NO is heavily involved in the immune response and is synthesized in large quantities by activated macrophages; it is called the "killer molecule" that helps destroy pathogenic microorganisms and tumor cells. NO is synthesized in the cell from the amino acid L-arginine, a process carried out by the enzyme nitric oxide synthase (iNOS for its acronym in English). (Cuellar et al., 2010)

NO is able to kill *Babesia* inside the infected erythrocytes, resulting in morphological structures known as "crisis forms" (Homer et al., 2000; Bock et al., 2004) that are nothing more than dead parasites inside the erythrocyte. However, it is not known with certainty whether this assumption is true. Thus the aim of the present study was to determine whether *L. casei* is capable of stimulating NO production in mouse macrophages which could control against the establishment of *Babesia* from a challenge infection in mice.

MATERIALS AND METHODS

Parasites

The *Babesia microti* Gray strain (Gleason et al., 1970) was used in the study. It has been maintained in liquid nitrogen and through passages in mice. All experimental infections were initiated with parasitized erythrocytes (PE) from donor NIH mice infected with frozen material. The infection dose used was 5 x 10^5 PE per mouse in 100 µl of saline inoculated by intraperitoneal (i.p.) route.

Animals

Parasite-free NIH female mice, 6 to 8 weeks old, with an average body weight of 30 g were obtained from the animal facility of the National School of Biological Sciences (National Polytechnic Institute) in Mexico City. Throughout the experimental period, commercial food and water were provided ad libitum.

Bacteria

The ATCC7469 strain of *Lactobacillus casei* was utilized (Bautista-Garfias et al., 1999). Organisms grown in MRS broth (Merck, Germany) at 37 °C for 18 h were washed twice with sterile phosphate-buffered saline (PBS) and resuspended in sterile PBS. The bacterial suspension was serially diluted tenfold with PBS, and the colony forming units (CFU's) were determined on MRS agar after incubation at 37 °C for 48 h. The standard inoculation used per mouse was of 1,8 x 10^9 CFU killed by heating (in boiling water during 30 min).

Percentage of parasitized erythrocytes (PPE)

PPE was estimated in each mouse in all groups (n = 6/group) by counting a total of 400 erythrocytes on Giemsa-stained smears of tail blood by optical microscopy. Readings were carried out in a blinded fashion. Individual percentage values were recorded from each mouse every day and used to obtain the mean group values.

Collection and culture of peritoneal macrophages

One day before the sacrifice of the mice were exposed to different treatments as indicated in Table 1. Six mice/group were sacrificed by cervical dislocation under sterile conditions, fixed with pins on a dissection tray and with the help of sterile scissors and forceps, the skin was separated from neck to tail. The sternum xiphoid process was located where 3,0 ml of sterile MEM-10% FBS were injected, through the diaphragm; then a peritoneal lavage was applied for 30-60 seconds. Afterwards, a needle was introduced to collect the peritoneal lavage fluid. The washing solution was decanted into a 15 ml conical tube and kept in an ice bath. After centrifugation at 450 g for 3 min, the supernatant was discarded and the cell pellet was suspended in 1 ml of cold culture medium MEM-10% FBS culture medium. Next, peritoneal macrophages were counted in a Neubauer chamber and adjusted to a final concentration of 1 x 10^6 cells/ml.

The macrophages were seeded in 24-well culture plates at 37 °C in the presence of 5% CO_2; after 24 hours the cells were observed to assure that they were not contaminated, using an inverted microscope (Nikon Eclipse TS 100). Then, 100 µl of the supernatants were collected and transferred to 96-well plates for NO quantitation. (Rojas et al., 1982)

Quantitation of nitric oxide

The assay is based on the reaction products of nitric oxide reduction (nitrite and nitrate)

Table 1. Experimental design

Group	Treatment (i.p) (day)	Infection (i) with 5 x 10^5 *B. microti*-parasitized erythrocytes (i.p.) per mouse *	Treatment assessment, day 1 (six mice in each group were sacrificed by cervical dislocation)
1. Control (n=6)	PBS (-1)	----	Nitric oxide roduction by peritoneal macrophages (NO PM)
2. Bm (n=12)	----------	i	(NO PM)
3. Lc (n=6)	*L. casei* (-1)	----	(NO PM)
4. Lc-Bm (n=12)	*L. casei* (-1)	i	(NO PM)

i.p. = intraperitoneal; PBS = 0,01M phosphate buffered saline pH 7,2; Bm = *Babesia microti*, Lc = *Lactobacillus casei*. *The percentage of parasitized erythrocytes was assessed by optical microscopy in Giemsa-stained blood smears in mice of groups Bm and Lc-Bm (6 mice/group) at days 0, 3, 7, 10, 12, 14, 18, 20, 22 and 26 after infection with *B. microti*.

with Griess reagent to form an azo compound that absorbs light at 540 nm (Giustarini et al., 2008). Griess reagent consisting of equal parts of 1% sulfanilamide in water and N-1-naphthylethylenediamine, 0,1% 2HCl in 2,5% phosphoric acid in water. A nitrite standard curve was performed from a stock solution of $NaNO_2$ 0,5mg/ml, using the following concentrations 50, 25, 12.5, 6.25, 3.125, 1.525, 0.7812, 0.3906 µg. For quantitation of NO, 100 µl of Griess reagent and 100 µl of culture supernatant in each well of 96-well plates were used. The mixtures were homogenized and then allowed to stand for 10 min at room temperature in the dark. The color development was measured in an ELISA reader (Multiskan plus) at 540 nm.

Statistical analysis

Data obtained were examined for differences in group means by one-way analysis of variance (GraphPad Prism 5) and Tukey's multiple-comparison test. Differences were considered as significant at a value $p < 0,01$.

RESULTS AND DISCUSSION

Percent of parasitized erythrocytes (PPE) curve observed in control mice after infection with *B. microti* was similar to curves previously obtained in previous experiments (Bautista et al., 2005; 2008); however, no parasites were detected in mice treated first with *L. casei* and then exposed to *B. microti* (Figure 1). This result indicates that the lactobacilli generated a strong protective innate immune response and corroborates previous findings in which very low percentages of parasitized erythrocytes were observed in mice treated with *L. casei* (Bautista et al., 2005). The high production of NO observed in mice infected with *B. microti* (Group Bm, Figure 2) might be related to the induction of NO production in macrophages directly by the parasite, as it has been previously demonstrated with *B. bovis* (Stich et al., 1998). It is also probable that these high levels of NO might be used by *Babesia* as a mechanism to evade of the host immune response. With regard to this, it has been shown that *B. bovis* merozoites invade host erythrocytes in vitro in around 10-30 seconds (Asada et al., 2012) and 70% of the merozoites of *B. divergens* infects erythrocytes in vitro in less than 45 seconds (Sun et al., 2011). Thus we speculate that in the inoculated group of Bm, *B. microti* accessed the circulatory torrent soon after challenge in the peritoneal cavity, but parasites also distracted the immune system by activating the peritoneal macrophages with a high production of NO in order to exhaust the early immune response. In contrast, NO produced by peritoneal macrophages previously activated by *L. casei* in mice of group Lc-Bm, was used by these cells to kill *Babesia* from a challenge infection in the mice of group Lc-Bm. (Figures 1 and 2)

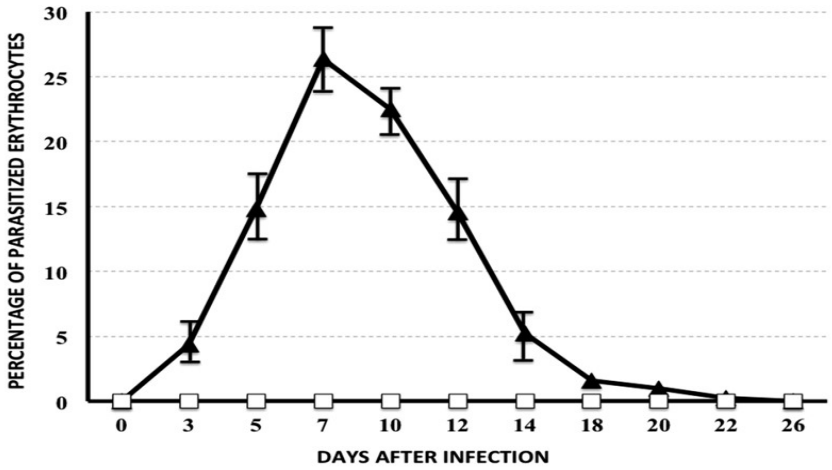

Figure 1. Percentage of parasitized erythrocytes (PPE) with *Babesia microti* in NIH mice after the intraperitoneal inoculation of 5 x 105 PE per mouse. Each point represents the mean + SEM of six mice. Black triangles, group of mice infected with *B. microti*; White squares, group of mice treated intraperitoneally with L. casei 24 hours before infection with *B. microti*.

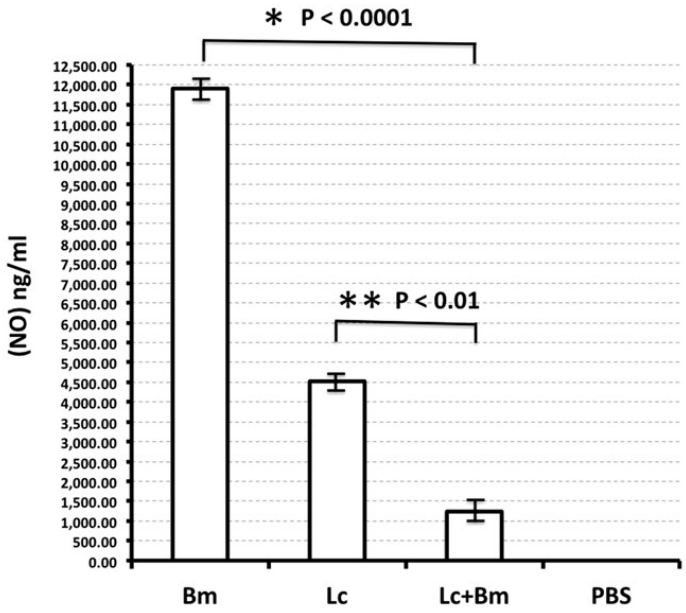

Figure 2. Nitric oxide (NO) (ng/ml) produced by peritoneal macrophages from mice with different treatments: Group PBS (control), Group Bm (*B. microti*), Group Lc (*L. casei*), Group Lc+Bm (*L. casei* + *B. microti*) after 24 hours of in vitro culture. Each point represents the mean + SEM of the cultures of peritoneal macrophages from six mice. * p < 0,0001; ** p < 0,01 (Tukey's test).

The significant low levels of NO in the group Lc-Bm as compared with those levels in the Lc group (Figure 2) suggest that NO killed *B. microti* after a challenge infection, since the parasite did not establish in the former group.

A fundamental part of the innate immune response in mice against acute infection with *Babesia*, is mediated by macrophages and NK cells, possibly through the early production of IL-2, INF-γ and induction of effector molecules such as nitric oxide derived from macrophages (Aguilar-Delfin et al., 2003). It was shown that in wild mice infected with *Babesia* WA1 had marked NO levels increase in serum, demonstrating that *Babesia* WA1 infection induces the early production of IL-12 and INF-γ. These cytokines have a role in the regulation of the innate immune response, which in turn activates macrophages to kill microorganisms, using NO as the effector destructive mechanism (Martínez-Gómez et al., 2006). Our results are similar to those obtained in previous studies in which macrophages of mice infected with different protozoan parasites such as *Trichomonas vaginalis* and *Plasmodium chabaudi*, showed increased expression of several inflammatory mediators, such as cytokines, chemokines and NO (Martínez-Gómez et al., 2006, Han et al., 2009). On the other hand, it has been demonstrated that some strains of *Lactobacillus*, such as *Lactobacillus casei* and *Lactobacillus rhamnosus*, effectively stimulate IL-12, thus inducing a dominant Th1 immune response, which includes macrophage activation, and enhancement of innate immune response. (Shida et al., 2009)

In support of our findings, it has been also shown that NO and its reactive nitrogen derivatives, produced by activated macrophages in vitro, inhibit intracellular protozoan parasites including *Leishmania major*, *Plasmodium falciparum* and *Babesia bovis*. (Green et al., 1990, 1990b; Johnson et al., 1996; Rockett et al., 1991; Taylor-Robinson, 1997; Taylor-Robinson et al., 1993)

CONCLUSIONS

L. casei activates macrophages when it is inoculated intraperitoneally in NIH mice and induces the production of NO in these cells. Thus, this metabolite might have a role killing *B. microti* from a challenge infection, and as a consequence the parasites did not multiply in the mouse erythrocytes.

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